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Biogas Digester Hydraulic Retention Time Affects Oxygen Consumption Patterns and Greenhouse Gas Emissions after Application of Digestate to Soil

Quan Van Nguyen,* Lars Stoumann Jensen, Roland Bol, Di Wu, Jin Mi Triolo, Ali Heidarzadeh Vazifehkhoran, and Sander Bruun

Abstract

Knowledge about environmental impacts associated with the application of anaerobic digestion residue to agricultural land is of interest owing to the rapid proliferation of biogas plants worldwide. However, virtually no information exists concerning how soil-emitted N_2O is affected by the feedstock hydraulic retention time (HRT) in the biogas digester. Here, the O_2 planar optode technique was used to visualize soil O_2 dynamics following the surface application of digestates of the codigestion of pig slurry and agro-industrial waste. We also used N_2O isotopomer analysis of soil-emitted N_2O to determine the N_2O production pathways, i.e., nitrification or denitrification. Two-dimensional images of soil O_2 indicated that anoxic and hypoxic conditions developed at 2.0- and 1.5-cm soil depth for soil amended with the digestate produced with 15-d (PO15) and 30-d (PO30) retention time, respectively. Total N_2O emissions were significantly lower for PO15 than PO30 due to the greater expansion of the anoxic zone, which enhanced N_2O reduction via complete denitrification. However, cumulative CO_2 emissions were not significantly different between PO15 and PO30 for the entire incubation period. During incubation, N_2O emissions came from both nitrification and denitrification in amended soils. Increasing the HRT of the biogas digester appears to induce significant N_2O emissions, but it is unlikely to affect the N_2O production pathways after application to soil.

Core Ideas

- O_2 planar optode images system and N_2O isotopomer analysis were deployed.
- O_2 consumption was greater for digestate with a hydraulic retention time of 15 d than 30 d.
- N_2O production was smaller for digestate with a 15-d hydraulic retention time.
- Lower N_2O emission for 15-d retention time digestate was due to higher complete denitrification.

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Reportedly, one of the main advantages of anaerobic digestion is the reduction in greenhouse gas emissions from the manure handling system, including storage and land application. Anaerobic digestion residues—digestates—have been reported to reduce nitrous oxide (N_2O) emissions after application to soils compared with undigested materials (Petersen et al., 1996; Köster et al., 2015). The main reason for this has been ascribed to the fact that easily degradable organic matter is degraded and transformed into methane (CH_4) during anaerobic digestion. However, there is also reason to believe that digestates could increase the production of N_2O after land application, depending on the properties of the digestates applied (Abubaker et al., 2013). The high water content of digestates may induce N_2O production immediately on application to soils because it limits atmospheric oxygen (O_2) diffusion into the soil, thus favoring denitrification (Firestone et al., 1989). At the same time, the residual content of easily degradable organic matter in digestates applied to soil increases O_2 consumption by soil respiration, which is driven by the supply of available carbon (C). This means that the application of digestate enhances the O_2 depletion zones where denitrifiers are able to produce N_2O (Bollmann and Conrad, 1998; Zhu et al., 2015).

The added organic C can also be used as an electron donor in the denitrification process (Tiedje, 1988). Thus, a reduction in organic matter in the digester will subsequently reduce the depletion area and propensity for denitrification, resulting in less N_2O formation from soil after digestate application. However, anaerobic digestion generally results in the digestates having a higher ammonia content, which can be oxidized by nitrification and produce N_2O as a by-product. This process requires O_2 for oxidation and thereby enhances O_2 depletion in the soil following application (Zhu et al., 2014). Hence, the effect of anaerobic digestion on N_2O emissions in the field is complex and depends

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Abbreviations: DM, dry matter; HRT, hydraulic retention time; PO15, hydraulic retention time of 15 d; PO30, hydraulic retention time of 30 d; SP, site preference; VSMOW, Vienna Standard Mean Ocean Water; WFPS, water-filled pore space.

on the properties of the digestate resulting from the different feedstocks used, the residence time in the digester, and interactions with climate and soil types.

These complex and interacting effects have so far been examined only to a limited extent. One of the few studies was undertaken by Clemens et al. (2006), who examined the direct linkage between hydraulic retention time (HRT) of the digester and N_2O emissions after land application of the digestate. They reported that increasing HRT from 0 to 29 and 59 d decreased emissions of N_2O. However, they did not study the underlying mechanisms in any detail; therefore, a more thorough understanding of these mechanisms is needed.

It is well documented that the supply of organic C and soil O_2 are some of the most important factors driving nitrogen (N) transformation processes (Tiedje, 1988; Bollmann and Conrad, 1998; Morley and Baggs, 2010). However, the way in which the distribution of O_2 in soil is influenced by the distribution and degradability of the organic matter applied, and in turn how this affects N_2O production, has not yet been fully investigated. A visualization of soil spatiotemporal O_2 dynamics by planar O_2 optodes has proved useful for enhancing understanding of the processes leading to N_2O emissions after application of organic materials (Zhu et al., 2014, 2015; Nguyen et al., 2017). Furthermore, N_2O isotopomer techniques have been used widely to investigate the source of N_2O production pathways (Toyoda and Yoshida, 1999; Bol et al., 2003b; Sutka et al., 2006; Jinuntuya-Nortman et al., 2008; Ostrom et al., 2010; Köster et al., 2013; Wu et al., 2017). More recently, a combination of these techniques was used to quantify the effects of cattle slurry application on soil O_2 distribution and N_2O emission pathways (Nguyen et al., 2017).

The objectives of this study were (i) to quantify the effect of HRT during biogas production on N_2O emissions after land application of digestates and (ii) to understand the role of O_2 dynamics for the determination of the N_2O production through different N_2O production pathways (i.e., bacterial denitrification and nitrification). It has been reported that the shorter HRT of biogas production led to a higher biodegradable fraction in digestates compared with digestates produced with a longer HRT (Vazifehkhoran and Triolo, 2015). Thus, the HRT of the biogas digesters could influence the O_2 consumption in soil after application of digestates to the soils. The resulting spatial and temporal distribution of soil O_2 consequently would affect N_2O formation and emission. Therefore, we hypothesized that digestates produced with a shorter HRT would induce larger anoxic zones and N_2O reduction compared with the digestates produced with a longer HRT when using the same feedstock.

Materials and Methods

Digestates and Soil

Digestates from the codigestion of pig slurry and agro-industrial waste were produced in 20-L continuously stirred anaerobic reactors operated with a HRT of 15 d (PO15) or 30 d (PO30). The feedstocks consisted of 75% pig slurry (wet weight) and 25% agro-industrial waste (a mixture of supermarket, brewery, and slaughterhouse waste). The temperature in the reactors was mesophilic with 37°C, and the digestates were collected daily and stored at −20°C for later use. The procedure used to produce the digestates was previously described in detail by Vazifehkhoran and Triolo (2015). Before application, the digestates were thawed and analyzed for physicochemical properties. Soil was collected from an experimental field in Foulum, Denmark, and was characterized as a sandy loam soil (79.1% total sand, 9.6% silt, 8.9% clay, 2.4% humus w/w). It was freshly sieved, field moist (<2 mm) just before the start of the experiment.

The dry matter contents of the digestates and the soil were determined by weight loss at 105°C for 24 h, and their total organic matter contents (loss on ignition) were determined after ignition at 550°C for 3 h. To determine the total organic N contents and total organic C of the digestates and the soil, the fresh samples of the digestates were dried at 70°C for 48 h, and samples of the soil were dried at 105°C for 24 h. These were then ground and analyzed using an elemental analyzer (vario PYRO cube; Elementar). The mineral N content of the digestates and the soil samples were measured after extraction with 1 M KCL (1:25 w/v). The extraction was then analyzed for NH_4^+ and NO_3^- concentrations by flow injection analysis (FIA-Series 8000; QuickChem). The total inorganic C content of the digestates was determined by measuring the carbon dioxide (CO_2) content released after acidification of 10 g fresh digestate samples with equivalent amounts of 1 M H_2SO_4 acid to reach pH 2.0 for each of the digestates using gas chromatography (450-GC; Bruker). The samples were kept in 0.75-L closed glass jars for 2 h. The physicochemical properties of the digestates and soil samples are presented in Table 1.

Incubation Experiment

An incubation experiment was conducted under laboratory conditions for 5 d to evaluate the dynamics of soil O_2 distribution and CO_2 and N_2O emissions after application of the digestates. The experiment consisted of three treatments: PO15, PO30, an untreated control soil (COTR). All treatments were performed with three replicates. The soil was packed in Plexiglas boxes (10 by 6 by 4 cm) fitted with planar O_2 optode foil on the front and rubber septa at the rear for gas sampling (Supplemental Fig. S1). The soil was compressed to a bulk density of 1.3 g cm^{-3}. Before digestate application, 17 mL of water was added to all optode chambers to achieve ~73% water-filled pore space (WFPS) and

Table 1. Properties of the soil and digestates used in the incubation experiment.

Measurements	Unit	Soil	PO15†	PO30†
Dry matter	g 100 g^{-1}	86.9	4.0	3.6
Organic matter (LOI‡)	% DM		64.8	66.2
Ammonium nitrogen (NH_4^+–N)	g kg^{-1} DM	2.2×10^{-3}	20.9	32.2
Nitrate (NO_3–N)	mg kg^{-1} DM	13.9	nil§	nil
Total organic N	% DM	0.25	2.63	2.58
Total organic C	% DM	2.0	36.0	35.2
Total inorganic C	g kg^{-1} DM		16.5	19.52
C/N ratio		8.2	13.7	13.7
pH		6.7	8.6	8.1

† Digestate of 75% pig slurry codigested with 25% agro-industrial waste (wet weight basis) at hydraulic retention times of 15 d (PO15) and 30 d (PO30).

‡ LOI: loss on ignition, 550°C oven for 3 h.

§ nil: negligible ~ 0 (mg kg^{-1} dry matter).

left for 48 h to equilibrate and for soil O_2 to stabilize. After this period, the digestates were applied to 50% of the soil surface at the rate corresponding to 100 kg NH_4^+ ha^{-1} (equivalent to 33.3 mg N kg^{-1} soil dry matter [DM]) for both PO15 and PO30 treatments, producing a final WFPS of 85% for all treatments, which was maintained throughout the incubation. The soil was left to incubate for 5 d at a temperature of 19 to 20°C. The detailed calculation of application rate is presented in the Supplemental Material (Supplemental Table S1).

Oxygen Optode and Soil Oxygen Imaging

During the incubation, images of the O_2 distribution were recorded every 30 min using the optode system (Supplemental Fig. S1). The system was previously described in detail by Zhu et al. (2015) and is based on the measurement principles described in Larsen et al. (2011). The optode system was calibrated using two calibration points of 0% and 100% of O_2 concentration in air-saturated water solution (Larsen et al., 2011). Soil O_2 content was calculated as the average O_2 content obtained for the entire optode window cross-sectional area (5 by 4 cm) and expressed as a percentage of air saturation using a free software ImageJ v. 1.50i (National Institutes of Health, 2016). Soil O_2 conditions were defined as anoxic, hypoxic, and oxic condition corresponding to <1%, 1 to 30%, and >30% air saturation, respectively (Zhu et al., 2014).

Trace Gas Emissions

Headspace gas sampling was performed 6, 24, 48, 72, and 96 h after digestate application to determine gas emissions and to perform N_2O isotopomer analysis. On each sampling occasion, the chamber was closed, and 5 mL headspace gas samples were taken every 20 min for 1 h, after which the chamber was opened again. Gas samples were injected into 3-mL pre-evacuated glass vials (Labco), and the concentrations of N_2O, CO_2 and CH_4 were determined by gas chromatography (GC-450; Bruker). Gas emission rates were calculated as the slope of a straight line fitted to the concentration of the gases in the headspace during the closed period. Cumulative gas emissions were calculated from the emission rates using the trapezoidal integration rule.

Nitrous Oxide Isotopomer and Source Partitioning

For N_2O isotopomer analysis, 25-mL gas samples were taken after a 1-h closed time on four occasions: 6, 24, 48, and 96 h after digestate application. By repeating this process, five 25-mL gas samples were collected and bulked into a 120-mL pre-evacuated crimped bottle to measure the N_2O isotopomer samples. The N_2O isotope signatures of soil-emitted N_2O gas samples, $\delta^{15}N^{\alpha}$, $\delta^{18}O$, the average $\delta^{15}N$ of the N_2O molecule ($\delta^{15}N^{bulk}$), were determined by measuring mass-to-charge ratio (*m/z*) 44, 45, 46 of intact N_2O^+ molecular ions, and *m/z* 30, 31 of NO^+ fragment ions using an isotope ratio mass spectrometer (IRMS-IsoPrime 100; Elementar Analysensysteme). The site preference (SP) value of soil-emitted N_2O is defined as $SP = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta}$, where $\delta^{15}N^{\beta}$ is the isotopic signature of $\delta^{15}N$ at the terminal position, which was calculated as $\delta^{15}N^{\beta} = 2\times\delta^{15}N^{bulk} - \delta^{15}N^{\alpha}$ (Toyoda and Yoshida, 1999). The soil emitted N_2O isotope signatures on day *t* (R_t) including $\delta^{18}O$, $\delta^{15}N$, and SP values were corrected with the reference N_2O isotope signatures of the ambient air in the laboratory using Eq. [1]. The measured $\delta^{18}O$ and $\delta^{15}N$ isotope signatures were expressed with respect to Vienna Standard Mean Ocean Water (VSMOW) and air standards, respectively. The correction and calibration of the measurements are detailed by Heil et al. (2015).

$$R_t = \frac{(R_{\text{sample}(t)} \times N_2O_{\text{sample}(t)} - R_{\text{air}} \times N_2O_{\text{air}})}{(N_2O_{\text{sample}(t)} - N_2O_{\text{air}})} \quad [1]$$

where N_2O_{air} and R_{air} are the average N_2O concentration and the corresponding $\delta^{18}O$, $\delta^{15}N$, and SP values of the laboratory air samples in the optode chambers before closure at t_0 (269 ± 5 ppb), and $N_2O_{sample(t)}$ and $R_{sample(t)}$ are the soil-derived N_2O concentration and their corresponding isotope signatures (i.e., either $\delta^{18}O$, $\delta^{15}N$, or SP) of the samples collected from optode chambers 40 min after closure (t_{40}) at day *t*.

The source partitioning of N_2O production was used to separate N_2O derived from either nitrification/fungal denitrification or bacterial denitrification. This was done by a two-end-member isotopic mass balance equation:

$$SP_t = SP_D \times f_D + SP_N \times f_N \quad [2]$$

where SP_t is the corrected site preference of soil-emitted N_2O obtained from Eq. [1], SP_D and SP_N are the SP values of N_2O produced by bacterial denitrification and bacterial nitrification or fungal denitrification in pure cultures, which ranges between −10 and 0‰ (average −5‰) and 33 to 37‰ (average 35‰), respectively (Toyoda et al., 2005; Sutka et al., 2006), and f_D and f_N are the portions of N_2O derived from bacterial denitrification and nitrification and/or fungal denitrification to total N_2O release ($f_D + f_N = 100\%$). Rearranging Eq. [2], the following equation was obtained:

$$f_D = [SP_t - SP_N\ (1 - f_N)] / SP_D \quad [3]$$

which can be used to calculate the contribution of bacterial denitrification to total N_2O production, f_D.

Statistical Analyses

All statistical analyses were performed using one-way analysis of variance (ANOVA) and Tukey multiple comparisons in RStudio (version 0.99.878) to determine the significant differences in the means of gas fluxes, cumulative gas emissions, N_2O isotopic signatures, SP values, and O_2 concentration between treatments. The significant differences were accepted at the level of probability of $P < 0.05$. Linear regression analyses were performed to examine the relationships between isotopic signatures of the soil-emitted N_2O between each treatment and for all treatments. Pearson correlation coefficients were obtained for the correlation between ^{18}O–N_2O and $^{15}N^{\alpha}$–N_2O.

Results and Discussion

Temporal and Spatial Distribution of Soil Oxygen

During the 48-h pre-incubation period, the soil O_2 content stabilized at approximately 75% air saturation throughout the soil cores of all treatments. However, the O_2 content was substantially depleted in the digestate-treated soils approximately 12 h

after digestate application (Fig. 1). In contrast, the O_2 content remained stable in the control soil after the addition of water. This is in line with Zhu et al. (2014), who observed rapid development of the anoxic and hypoxic zones within the first 5 h of pig manure application to soil, either in layers or mixed into the soil. Similarly, Nguyen et al. (2017) applied cattle slurry to the soil surface and observed a depletion zone developing beneath the application area after just 2 h, with the most extensive depletion between 18 and 24 h.

The recorded depletion zone clearly covered a larger area for PO15 than for PO30 (Fig. 1 and Supplemental Videos S2–S3). Within the first 24 h, anoxic zones developed rapidly for PO15 and peaked on approximately 30% of the total 4- by 6-cm image area, as opposed to only 10% for PO30 (Supplemental Fig. S2). The anoxic area that developed in the current study was much less extensive than in the study by Zhu et al. (2014) due to lower biodegradable organic matter in the digestate compared with undigested material. Hypoxic zones were similar in size for PO15 and PO30 within the first 24 h after digestates application, but it was much larger and remained the predominant soil condition in PO15 from 24 to 48 h compared with PO30, where the hypoxic zone was quickly reduced during this period.

The fact that the O_2 depletion zones were much larger for PO15 than PO30 suggests that more easily biodegradable organic C in the PO15 digestate still remained as a result of the shorter 15-d HRT. This resulted in a higher demand for O_2 after application compared with that of the PO30 digestate produced with 30-d HRT. The obvious explanation for this is that during anaerobic digestion, easily degradable organic C in the substrates is gradually transformed into CH_4, and this process works more efficiently when the substrates have a longer retention time in biogas digesters (Vazifehkhoran and Triolo, 2015; Fitamo et al., 2016). The presence of more biodegradable organic matter applied in the PO15 treatment—a total organic C of 478.7 mg kg^{-1} soil dry weight for PO15 compared with 388.2 mg kg^{-1} for PO30 (Supplemental Table S2)—therefore stimulated greater microbial activity, which is the main reason for the more severe depletion of O_2 in the PO15 treatment. Another possible explanation for the stronger depletion of the O_2 content in the PO15 treatment compared with the PO30 treatment could be higher consumption by nitrification. However, since the application of the digestates was based on the same total amount of NH_4^+ for PO15 and PO30, it was assumed that O_2 consumption for nitrification would be similar for both PO15 and PO30.

The O_2 images showed that the O_2 depletion zones developed immediately beneath the area where the digestates were applied and expanded both downward and horizontally in both directions (Fig. 1). The stronger O_2 depletion in the upper soil layers (0–2 cm) was caused by their proximity to the applied manure, with most of the O_2 consumption occurring in the upper layer of soil through which dissolved organic matter percolates. This is supported by Bol et al. (2003a), who reported that easily biodegradable, cattle manure-derived C is likely to be the main source of C for soil microbial respiration within the first 48 h in the top 2-cm soil layer after application. Recently, Nguyen et al. (2017) also observed a depletion zone in the upper 1.5 cm after application of cattle slurry on the soil surface. However, in that study, there was a tendency for a less intensive depletion of soil O_2 below the soil surface, presumably because of a higher influx of O_2 from the surface. It is not clear why this influx is more restricted in the current study, but it could be related to the physical properties of the soil (e.g., soil bulk density, pore size, and soil WPFS) and their interaction with soil biological processes (Balaine et al., 2013, 2016; Owens et al., 2017). Soil porosity and macroporosity declines with increasing soil bulk density (Balaine et al., 2013). Therefore, the higher soil bulk density of 1.3 g cm^{-3} for the present study could lead to a lower O_2 diffusion from the

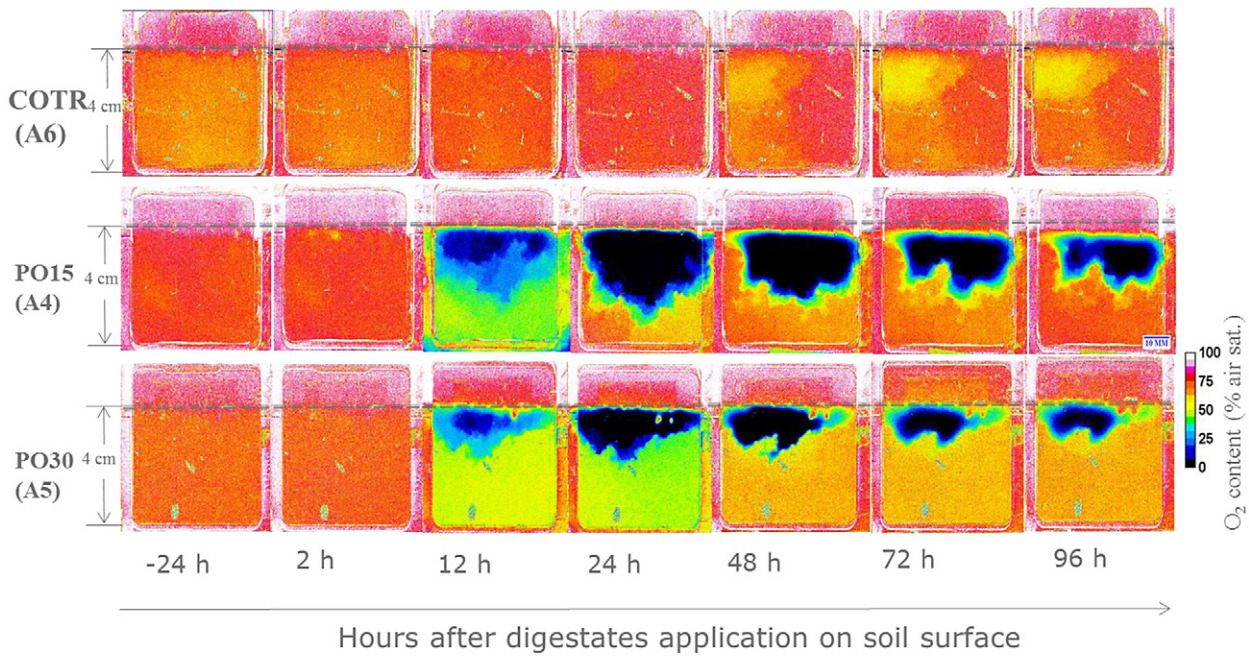

Fig. 1. Selected two-dimensional images of soil O_2 distribution (% air saturation) at different times after digestate application for a representative chamber (1 replicate) of the control (COTR, Supplemental Video S1), digestate with a 15-d retention time (PO15, Supplemental Video S2) and a 30-d retention time (PO30, Supplemental Video S3). For others replicates during the incubation, see Supplemental Videos S4–S9).

soil surface into the deeper soil layers compared with 1.0 g cm^{-3} in Nguyen et al. (2017). Also, the relatively high dry matter content of the cattle slurry (11.5% w/w) in their study compared with that of the digestates (3.6–4.0% w/w) in the present study is likely to limit the infiltration of easily biodegradable, soluble or fine particulates into the soil, thereby causing fewer O_2 depletion zones beneath the application areas.

It is apparent from the optode images that O_2 from the headspace of the chambers diffused into the soil through the surface areas on which the digestates were not applied (Fig. 1). This clearly diminished the O_2 depletion zones for both the PO15 and the PO30 treatments in the upper soil layer and led to the increase in soil O_2 content outside the application area (Supplemental Videos S2–S3). This implies that O_2 consumption was lower in the areas without digestate application than in the application areas. After 48 h, soil O_2 increased throughout the soil cores for both PO15 and PO30 (Fig. 1, Supplemental Fig. S2). This demonstrates that the demand for O_2 for microbial respiration and nitrification decreased, presumably because the applied active labile C and N had been consumed by this time and the influx of O_2 from ambient air was greater than O_2 consumption. Although the soil water content was as high as 85% WFPS in the control soil, oxic conditions dominated in the soil cores throughout the incubation period. This was due to the low O_2 demand for both microbial respiration and ammonium oxidation since soil organic C (2% DM) and soil NH_4^+ (2.2 mg kg^{-1} DM) were relatively limited (Table 1).

Greenhouse Gas Emissions

During the incubation, CH_4 emissions from all treatments remained at a very low level (<0.1 μg kg^{-1} soil h^{-1}); however, the CO_2 and N_2O emissions from digestate-treated soils were substantially higher than from the control soil (Fig. 2A–B). The CH_4 emissions (data not shown) were comparable to previous incubation studies for digestate soil amendments (Odlare et al., 2012; Abubaker et al., 2013). In both digestate soil amendments, CO_2 emissions already occurred at a considerable rate (~1.2 mg C kg^{-1} soil h^{-1}) at 6 h after digestate application but then declined toward the end of the incubation, whereas CO_2 emissions from the control soil were negligible (~0.05 mg C kg^{-1} soil h^{-1}) and relatively constant. This is in line with previous studies (Köster et al., 2011, 2015; Alburquerque et al., 2012), which reported a pronounced initial peak of CO_2 evolution during the initial 24 h after application of a digestate from the codigestion of pig slurry and/or cattle manure with agro-industrial wastes with HRT about 56 d. It is assumed that the main difference between the digestates with retention times of 15 and 30 d is the content of labile biodegradable C, with less difference in the recalcitrant organic C content. Thus, since most of the labile C was respired by aerobic microbes at the start of the incubation, the recalcitrant organic C was left behind in the soil and decomposed at slower rates in both PO15 and PO30, resulting in the decline in CO_2 evolution and lower demand for O_2 consumption for soil respiration. Although the total organic C applied in PO15 was much higher than in PO30 (Supplemental Table S2), the total C mineralization (% CO_2–C released of C added) over the 5-d incubation for PO15 was not significantly higher than that of PO30 (Supplemental Table S3). This could be due to the higher anoxic conditions developing in PO15, which limited the magnitude of soil aerobic respiration and reduced the magnitude of difference in CO_2 evolution compared with PO30.

For N_2O emissions, both digestate treatments induced a significant N_2O flux compared with the control, but the magnitude of this stimulation varied between the digested materials. The N_2O emissions from the control were negligible (~0.01 μg N kg^{-1} soil h^{-1}) throughout the incubation, as expected, due to the low NH_4^+ content in the soil and hence limited nitrification. Also, the high O_2 content throughout the soil matrix (75% air saturation) limited denitrification (Smith and Tiedje, 1979). However, for the digestate-amended soils, both processes could have occurred where the presence of added NH_4^+ and organic C caused considerably higher N_2O emissions (Fig. 2A, C).

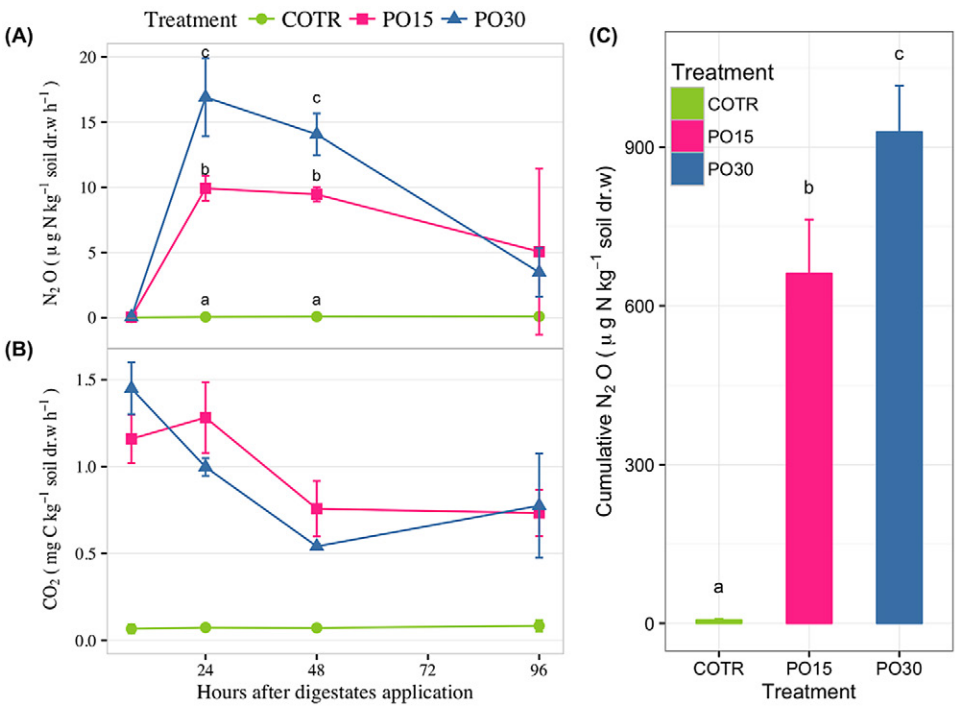

Fig. 2. Gas fluxes (A) N_2O, (B) CO_2, and (C) total cumulative N_2O emissions over the entire 5-d incubation period for the control (COTR), soil treated with digestate with a 15-d retention time (PO15), and soil treated with digestate with a 30-d retention time (PO30). Letters indicate significant differences with $P < 0.05$; error bars indicate standard deviation of the mean values.

Higher N_2O emissions after the application of digestates to soils compared with the unfertilized control soils were previously reported (Köster et al., 2011; Rodhe et al., 2012; Abubaker et al., 2013). Our study showed that N_2O emissions were still very low (<0.1 μg N kg^{-1} soil h^{-1}) in both PO15 and PO30 6 h after application, when oxic conditions remained the dominant condition in all treatments (Supplemental Fig. S2). However, after this, N_2O increased dramatically and peaked after 24 h for both digestate treatments, simultaneously with the strongest O_2 depletion in the soils. This initial peak of N_2O was previously reported after slurry application (Petersen et al., 1996, 2016; Nguyen et al., 2017) and digestate application (Köster et al., 2011, 2015; Abubaker et al., 2013).

The N_2O flux was nearly twice as high in PO30 as in PO15 at its peak and remained greater over the next few days, resulting in significantly higher cumulative N_2O emissions over the entire 5-d incubation period for PO30 than for PO15. This could suggest that the higher O_2 consumption in PO15 during the initial 48 h, which was due to the higher respiration activities as previously discussed, significantly decreased soil-emitted N_2O. The most plausible explanation for this is that the widespread anoxia developing in the PO15 treatment within the first 24 h after application did more to stimulate complete denitrification, where N_2O is reduced to N_2. In contrast, this step did not occur to the same extent in the PO30 treatment because of the relatively lower anoxia areas compared with PO15. This explanation is partly supported by Miller et al. (2009), who reported the negative correlation between respiration rate and N_2O molar ratio, that is, $N_2O/(N_2O + N_2)$, in liquid manure-amended soils. These authors therefore proposed that a higher C substrate availability in soil enhances the reduction of N_2O to N_2.

The complete denitrification step was previously observed with the high water content in digestate-amended soil where no immediate N_2O peak was reported after the application of anaerobically digested cattle manure (Köster et al., 2015), although in their study, the relatively high initial soil nitrate (31 mg kg^{-1} soil DM) at 90% WFPS soil moisture level contents usually resulted in N_2O peaking shortly after the application of digestate. In the present study, the application of higher labile C clearly increased demand for terminal electron acceptors (NO_3^-) in PO15 relative to PO30. Consequently, readily available soil NO_3^- (13.9 mg kg^{-1} soil DM) was used preferentially, and immediately, within the initial 24 h to produce N_2O (Cho et al., 1997) in the digestate-amended soils. However, the anoxia was approximately 10 and 15% of the total area for the PO30 and PO15, respectively, at the peak of N_2O emissions by 24 h, whereas the hypoxia fraction was close to 60% of the total area for both PO15 and PO30 (Supplemental Fig. S2). This condition was not optimum for the dominance of complete denitrification in digestate-amended soils.

The N_2O emission rate gradually reduced over the following days and approached the background level after 96 h for both PO30 and PO15, with oxic conditions returning in >80% of the total soil area (Fig. 1, Supplemental Fig. S2). Thus, at this point, it was concluded that either a lack of electron donor (digestate-derived organic C) and electron acceptor (soil NO_3^-) supply for denitrification or the dominance of oxic conditions in soils inhibited N_2O production from the denitrification process.

Nitrous Oxide Isotopomer Signatures and Source Partitioning

Nitrous oxide isotopomer signatures showed that the SP values, $\delta^{18}O$ (VSMOW), and $\delta^{15}N^{\alpha}$ of soil-emitted N_2O for digestate-treated soils fluctuated, whereas the values were almost constant for the untreated control soil during the incubation period (Fig. 3). For the control soil, N_2O emissions were very low; hence, the SP values of emitted N_2O for the control were similar to those of the ambient air, approximately 17‰ (Yoshida and Toyoda, 2000). In contrast, the SP values of digestate-treated soils clearly increased from 17 to 24‰ in both digestate treatments during the initial 24 h, thereafter gradually declining over the next few days (Fig. 3B).

The SP values of soil-emitted N_2O from PO15 and PO30 at most of the sampling times, except at 24 h, were within the range

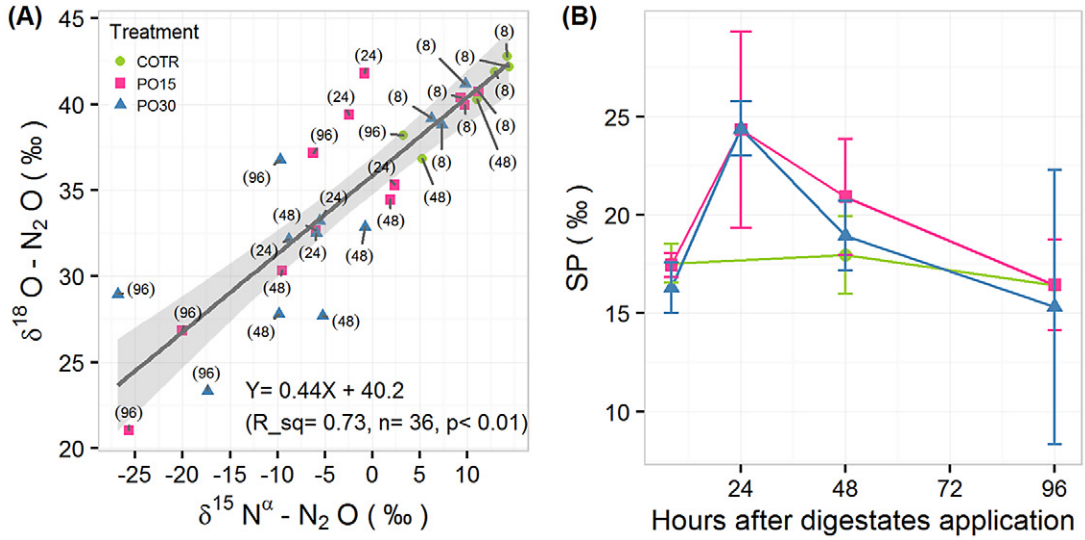

Fig. 3. (A) Correlation between $\delta^{18}O$-N_2O (Vienna Standard Mean Ocean Water) and $\delta^{15}N^{\alpha}$-N_2O for treatment control (COTR), digestates with a 15-d retention time (PO15), and digestates with a 30-d retention time (PO30). Data represent each single measurement for replicates. Numbers in parentheses indicate sampling time (hours after application). The individual correlation between the isotopic signatures within each treatment is shown in Supplemental Table S4. (B) Site preference (SP) values of soil-emitted N_2O during the entire 5-d incubation period.

of predominant bacterial denitrification-produced N_2O in soil environments (Bol et al., 2003b; Well et al., 2006; Opdyke et al., 2009; Köster et al., 2011, 2015). Within the initial hours and/or days after slurry-related material application to soils, the initial peak of N_2O has often been observed and attributed to denitrification of the soil nitrate following the addition of active C and N. This stimulates activities of nitrifiers and denitrifiers at the manure "hot-spot," thus creating O_2 depletion zones and inducing N_2O production (Paul et al., 1993; Petersen et al., 1996; Meyer et al., 2002; Köster et al., 2011; Markfoged et al., 2011; Abubaker et al., 2013). However, by 24 h, the highest SP values for both PO15 and PO30 were around 25‰, at the peak of N_2O emissions, corresponding to approximately only 26% of the estimated N_2O originating from bacterial denitrification according to the two-end-member equation (Supplemental Table S5). A likely explanation for these relatively high SP values is the isotope fractionation effects of the N_2O reduction via complete denitrification (Ostrom et al., 2007; Jinuntuya-Nortman et al., 2008; Well and Flessa, 2009; Köster et al., 2013). Thus, the estimation of N_2O derived from bacterial denitrification based on a two-end-member calculation using Eq. [3] can be underestimated (Wu et al., 2016).

Against this backdrop, it could be expected that complete denitrification occurred in both PO15 and PO30. However, the extent of this could not be quantified since N_2 emissions were not measured in the present study. It has been reported that the SP fractionation factor of N_2O reduction values ranges from −16.4 to −1.9‰ (Well and Flessa, 2009). Taking this variation into account, the increasing SP values in the present study are a good indication that N_2O reduction via complete denitrification occurred within the initial 24 h after digestate application. This is also supported by Köster et al. (2015), who reported that the $N_2O/(N_2O + N_2)$ product ratio was close to zero during the initial period (<24 h) after either cattle slurry or its digestate were applied to soils, and this ratio then increased in the later stages.

Alternatively, Nguyen et al. (2017) proposed that an early peak of N_2O occurring within the initial 24 h after cattle slurry applied on the soil surface could be associated with fungal denitrification in an acidic grassland soil (pH 5.7). Although fungal denitrification was also reported as a major source of N_2O production at the relative neutral soil pH 6.3 (Laughlin and Stevens, 2002), the soil moisture content in their study was relatively low at 65% WFPS, which significantly influenced the contribution from fungal denitrification. Chen et al. (2015) demonstrated that the fungal-to-bacterial contribution ratio at high WFPS such as 85 and 90% were significantly lower than that of 65 and 75% WFPS. Therefore, in the present study, the higher soil pH 6.7 and soil moisture content (85% WFPS) were not likely optimal conditions for fungal denitrification. In addition, the potential for fungal denitrification to produce N_2O remains in soils with enhanced organic C (Laughlin and Stevens, 2002; Köster et al., 2015) and under subanoxic conditions (Jirout et al., 2013; Chen et al., 2015; Lewicka-Szczebak et al., 2016). In the present study, the O_2 optode images showed that anoxic and subanoxic conditions (hypoxia) dominated within the first 24 h; thus, fungal denitrification could play a role in N_2O production during this period. The contribution from fungal denitrification could be also considerable after the initial 24 h in the PO15 treatment since the hypoxia remained widespread in PO15 until 48 h (Supplemental Fig. S2). However, the SP values had declined substantially between 24 h and 48 h (Fig. 3B), indicating that fungal denitrification was unlikely the dominating source of N_2O production after the first 24 h in the present study. Therefore, the N_2O fluxes from fungal denitrification was possibly important during the first 24 h but unlikely to be the dominant source for the entire course of the incubation under the present experimental setup.

After 24 h, since the N and O isotopic signatures of soil-emitted N_2O were gradually depleted toward the end of the incubation from 0 to −25‰ and from 40 to 30‰, respectively, the dominant source of N_2O production appeared to be shifting toward bacterial denitrification (Mandernack et al., 2000). This was evidenced by the significant positive correlation between $\delta^{18}O$-N_2O and $\delta^{15}N^{\alpha}$-N_2O with a slope of 0.44 (Fig. 3A, Supplemental Table S4), which is typical for systems in which N_2O is produced and consumed simultaneously (Mandernack et al., 2000; Ostrom et al., 2007). These observations were in accordance with the decline of SP values after 24 h toward the end of the incubation.

The estimated contribution of bacterial denitrification to N_2O increased from 26 to 35 and 46% for PO15 and from 26 to 45 and 55% for PO30 between 24, 48, and 96 h after application, respectively (Supplemental Table S5). This seemingly contrasts with the fact that significantly lower CO_2 fluxes observed compared with the fluxes within the initial 24 h is indicating the depletion of the electron donor (organic C) in the later phases (48 h, 96 h). This could diminish the extent of denitrification in both digestate treatments. It has been reported that aerobic respiration is positively correlated with denitrifier community abundance in slurry-amended soils (Miller et al., 2009; Köster et al., 2015). Furthermore, either the development of oxic conditions toward the end of the experiment after 48 h, which could inhibit NO_3^- reduction, or limited supply of soil NO_3^- presumably resulted in a reduction in denitrification in the later phase of the incubation. In addition, higher labile C applied to the soil for PO15 provided more available energy for denitrifier organisms compared with PO30, leading to an expectation of higher denitrification in PO15 than in PO30. However, the estimated bacterial denitrification for these two treatments on each sampling day were similar, indicating that denitrification was unlikely to be limited by the C supply in the digestate-amended soils. Consequently, the effect of HRT on the source partitioning of soil-emitted N_2O was not significant in the present study.

During the 5-d incubation period, the contribution of either nitrification and/or fungal denitrification or bacterial denitrification to N_2O production from digestate-treated soils was not influenced by HRT. The estimation of sources of N_2O production based on the two-end-member of the SP values indicated that nitrification appeared to be the dominant N_2O production contributor in the digestate treatments (Supplemental Table S5), even though denitrification was expected to be the main production pathway at a high soil moisture content in this experimental setup. This is partly because of the relatively low emission rates measured during the incubation for both digestate treatments compared with the peak after 24 h, which resulted in a small contribution of bacterial denitrification to the total cumulative N_2O for the entire incubation period.

Conclusions

Soil O_2 content was substantially depleted in the 0- to 2-cm soil depth after surface application of the digestates. Higher O_2 consumption in the PO15 compared with PO30 treatment resulted in larger anoxic and hypoxic zones for at least 48 h after application. The larger area of anoxia led to an apparently more complete reduction of NO_3^- to N_2 in the PO15 treatment, thus reducing N_2O emissions. The longer hydraulic retention time of digestate induced significantly higher N_2O emissions after soil application, probably due to lower microbial O_2 consumption and hence the lesser extent of anoxia. The N_2O source partitioning was not significantly affected by the biogas digester retention time ($P > 0.05$). During the incubation, the N_2O isotopomer signatures indicated that both denitrification and nitrification apparently contributed to produce N_2O emission for both digestates. The isotopic fractionation during the reduction of N_2O to N_2 in the initial 24 h may have led to some underestimation of N_2O produced by bacterial denitrification.

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